CHAPTER NINE

Response of Tannins and Phenolics of Willows to Simulated Herbivory, Water Dynamics, and Ambient Elk Herbivory on the Elk Winter Range of Rocky Mountain National Park

By

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Abstract. Plant secondary metabolites, including phenolics and tannins, function as defenses against natural enemies, and link several ecosystem processes through diverse mechanisms. We reviewed and summarized literature regarding the response of natural product chemistry in willows (*Salix* spp.) to grazing on sites that vary in their ability to support willow growth. This summary provided a conceptual model that was used to develop hypotheses regarding the effects that different site conditions might have on the ability of willows to respond to grazing by elk and mechanical clipping. Willow dynamics in response to elk herbivory on the elk winter range of Rocky Mountain National Park (RMNP), Colorado has been a topic of concern for most of this century. We examined the secondary metabolite responses of three willow species on the RMNP winter range under a number of different site conditions and grazing treatments. Sites were characterized alternatively as either: (1) wet or dry, based on depth to water table; (2) tall, short-watered, or short-control based on willow growth, presence of beaver activity, and water amendments made through installation of check dams; and (3) grazed, clipped, or ungrazed based on imposed herbivory treatments. Studies were conducted from 1995–1999 in exclosures erected in 1994. Additionally, we examined responses of willows protected for 30–35 years to varying levels of mechanical clipping (4) [0%, 50%, 100%] and compared to willows exposed to ambient elk herbivory for 30–35 years.

Willow species differed in phenolic and tannin production in most comparisons with phenolic production highest in summer leaves and twigs for *S. planifolia* followed by *S. monticola* and then *S. geyeriana*. *S. monticola* had higher tannin concentration than *S. planifolia*, with *S. geyeriana* producing the least. Phenolic production in twigs with leaves was significantly greater than that of twigs collected in the winter. Tannin production in willows at the tall sites in 1995 was higher compared to the short-control and short-watered sites. Tall willows from *S. geyeriana* and *S. monticola* in January 1999 had higher tannin concentrations compared to willows on the short-control sites, but tall willows did not differ from those at the short-watered sites.

We expected that willows growing on favorable habitats would respond to clipping and elk grazing by increasing their phenolic and tannin concentration. Few patterns were found that were consistent with the predicted results. However, willows growing on sites that were delineated by the presence of standing water and beaver activity did

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However, willows growing on sites that were delineated by the presence of standing water and beaver activity did have higher concentrations of phenolics and tannins compared to willows on sites with no standing water or recent beaver activity. Additionally, few significant increases in phenolic and tannin concentration were noted in the clipping and grazing treatments. These data, along with the lack of response in willows subjected to natural grazing levels, suggest that elk grazing has adversely affected the ability of willows to respond to grazing.

Tannins and phenolics tended to increase in the 50% clipped treatment in the 35-year-old exclosure. This suggests that the heavier 75% clipping level may have prevented willow response in the 4-year-old exclosure experiments. It was expected that willows grazed at ambient levels by elk outside the exclosure would also respond to elk grazing by increasing their secondary metabolite production such as that observed for the 50% clipping (ambient level of removal was 33% of current annual growth). Since grazed willows did not respond similarly, this suggests that elk have adversely affected willow physiology. Overall, the pattern noted among treatments in the 35-year-old exclosure represents the expected response of willows that are growing in a favorable environment. The lack of response in terms of increased secondary metabolites of heavily grazed willows could lead to greater palatability, and thus evermore increased grazing pressure on these plants.

Keywords: Elk, herbivory, phenolics, secondary metabolites, tannins, willow.

Introduction

At one time, secondary metabolites--which include the phenolics and tannins of willow (*Salix* spp.)--were thought to be waste products that might confer advantage to plants as antiherbivore and antimicrobial agents. Now they are known to function as defenses against natural enemies, and to link several ecosystem processes through diverse mechanisms (Cates 1996; Schimel et al. 1996). Tannins affect nutritional quality by binding with several primary metabolites (e.g., nitrogen-containing compounds like proteins, amino acids, DNA, RNA) often making them difficult to digest. Low molecular weight phenolics have been suggested to be toxic and/or act as feeding deterrents (Robbins 1993). Of these two, low molecular weight phenolics may play the major role in deterring ungulate grazing since some large animal herbivores may have evolved mechanisms that make tannins less effective as defenses (Robbins et al. 1987). For willows inhabiting sites favorable for growth, the challenge from tissue removal by herbivores and infection from pathogens normally results in an induced increase in these chemicals in remaining and new leaf and stem tissues (Karban and Baldwin 1997). In willows, where conditions for growth are unfavorable, plants may not be able to respond by increasing their production of defensive chemicals when exposed to natural enemies and simulated herbivory (Table 1). Comparing the response of tall willows versus short willows and willows growing on wetter sites with those on drier sites may aid in understanding the effects of abiotic conditions and grazing on willow physiology and growth (Johnston et al. 1993).

Because secondary metabolites are known to influence soil, litter, nutrient, and microbial processes, any change in the ability of willows to produce these chemicals may affect plant community dynamics. Low molecular weight phenolics serve as carbon sources for carbon-limited soil microbes, enhance immobilization of nutrients (Schimel et al. 1996), and act as inducers of microbial-higher plant interactions (Phillips 1992). Tannins, low molecular weight phenolics, and terpenes in litter have been shown to inhibit nitrification (Thibault et al. 1982; Baldwin et al. 1983; White 1986; Schimel et al. 1996). Tannins also inhibit microbial activity (Field and Lettinga 1992) and affect mineralization rates (Benoit et al. 1968; Schimel et al. 1996), but the mechanisms of action are unclear. Tannins bind to proteins that may make soil nitrogen (N) less available to microbes and higher plants; they may also bind to microbial exoenzymes leading to inhibition in the breakdown of tannin polymers (Benoit and Starkey 1968). Terpenes can act as toxic agents (White 1986) or as carbon sources for microbial growth, thereby tying up N through immobilization (Bremner and McCarty 1988). Based on these complex interactions, we have taken a broad conceptual approach in our studies of the effects of herbivory on willow secondary chemicals in litter and nutrient cycling (Table 1).

Table 1. Verified and predicted parameters describing sites favorable and unfavorable for willow growth, and willow and habitat responses on these sites, in the northern winter range of Yellowstone National Park, Wyoming. Taken from Cates et al. (1999).

Parameter/Response	Site	
	Unfavorable	Favorable
Parameters		
Precipitation[1-4, 8]	Less, drier winters	Higher, wetter
Temperature[1-4, 8]	Warmer	Cooler
Water tables[1-3]	Lower	Perennially higher
Streambanks[1-3]	Prevalent downcutting	Less downcutting, vegetated
Flooding[1-3]	Common, extensive	Infrequent, reduced intensity
Streams[6, 7]	Single or few streams, narrow streams; increased flow	Network of braided, expounded streams, ponds; impounded water, high sediment organic matter
Beaver[3, 5, 6]	Few to none; loss of food supply	Common; food supply abundant (aspen, willow)
Willow Response		
Production[1-3]	Reduced; negative gain in CAG	Net increase, vigorous gain in CAG
Recruitment[1-3]	Reduced to nonexistent	Seed productivity high, asexual
Leader length and weight[1-3]	Short, lighter	Longer, heavier
Plant height[1-3]	Reduced in stature	Able to grow beyond reach of elk
Browsing effect on growth form[2]	Morphological juvenility response; reduced physiological response	Morphological and physiological juvenility
Defense response	Reduced or none	Induced response vigorous
Habitat		
Nutrients[6, 9]	Reduced input of N	Enhanced N availability
Aquatic invertebrates[6, 7]	Shredders, scrapers; biomass less	Collectors, predators; greater biomass
Carbon[6, 7]	Low carbon input	High carbon input and processing
Litter	More recalcitrant; slower processing	More rapidly processed
Plant community[2, 7, 8]	Grass, sedges, conifer encroachment, dryland shrubs	Mosaic of riparian vegetation; willow, aspen maintained

[1]Singer et al. (1998)
[2]Singer et al. (1994)
[3]Singer and Cates (1995)
[4]Balling et al. (1992a,b)
[5]Consolo-Murphy and Hanson (1993)
[6]Naiman et al. (1988)
[7]Naiman and Melillo (1984)
[8]Houston (1982)
[9]Jonas (1955)

Willow and aspen dynamics on the elk winter range of Rocky Mountain National Park (RMNP), Colorado has been a topic of concern and discussion during most of this century (Hess 1993). Over a 25-year period (1968–1992) after elk were released from human controls and when elk increased dramatically in the park, park biologist David Stevens concluded that increased grazing may be the most important cause of willow decline in some areas of the winter range (D. Stevens, National Park Service, unpublished annual reports 1969–1992). Stevens suggested that under current high levels of elk herbivory, willow survival might occur only in habitats capable of sustaining sufficient growth. Changes in willow cover over the 25-year period from 1968 to 1992 on six sites containing marked willows, were inconclusive, with one-third of the sites showing willow decline, one-third showing stable cover, and one-third showing increasing willow cover (Zeigenfuss et al. 1999). Zeigenfuss et al. (1999) noted that this sample size of willow plots was very small (n = 6) and inference was limited in this study. However, analysis of willow cover across the entire winter range landscape using aerial photo interpretation indicates that the willow decline on the entire winter range since 1946 has been about 19–21% (Peinetti et al., this volume).

Stevens (National Park Service, unpublished annual report [1991]) also suggested that beaver eliminated willow at pond sites and then abandoned these sites on the winter range. He noted that the loss of beaver might be related to a drop in the water table such that many sites on the winter range may not be able to support heavily grazed willow. Beaver populations have declined 94% over the last 60 years on the winter range (Zeigenfuss et al., this volume). Consequently, elk herbivory, hydrological considerations, and beaver may be important and interactive factors that affect willow dynamics in various ways. With these scenarios in mind, we reviewed the literature in an effort to develop a conceptual model delineating the response of natural product chemistry of willows to grazing on sites that may vary in growing conditions (Table 1).

Willows growing under favorable abiotic conditions appear to be able to withstand moderate levels of grazing by ungulates (Table 1, references therein). Under these conditions willows add new tissues, reproduce, and respond to grazing with induced defenses while rapid vertical growth rates allow at least a portion of the plant to grow beyond the reach of ungulates. Sites characterized by high water tables and favorable nutrient dynamics appear to be favorable to the vigorous growth of willows (Table 1). These dynamics appear to be greatly facilitated by the presence of beaver. Alternatively, sites characterized by lower water tables, relatively narrow, fast moving streams, lack of suitable litter and nutrient dynamics to sustain willow growth, and fewer beaver, seem to result in conditions unfavorable to healthy riparian willow communities (Table 1).

Based on these considerations, the following predictions were developed. For sites at RMNP that appear to have more favorable growing conditions for willows (e.g., tall willow sites, or sites with higher water table levels), the expectation was that willows inside exclosures that were clipped at levels similar to that for elk grazing would respond by increasing their phenolic and tannin concentration. On sites less favorable to willow growth (e.g., short willow sites, or sites with lower water tables), the expectation was that willows would not respond to clipping when compared to the unclipped, ungrazed control. Tall willow were predicted to respond differently than short willows in that the tall structure was demonstrated on this winter range to be largely a function of not only lower levels of ambient herbivory (Singer et al., this volume), but also good growing conditions. Tall willows on good sites were suspected to have deeper rooting zones than heavily browsed willows in locations further from streams, based on different isotopic signatures (Menezes et al., this volume). In other words, even though height of tall browsed willow was a product of several multiplicative factors including level of elk herbivory and site conditions, we expected tall willows to respond to the herbivory treatments differently than short, browsed willows.

Protection from grazing for long periods was also predicted to result in greater ability to respond with an increase in these natural products to moderate clipping. However, willows would not be able to respond by increasing phenolic and tannin concentration when subjected to either clipping 100% of new growth, or the high level of elk grazing observed on short willows on some parts of the winter range. For willows that were not protected from grazing prior to clipping treatments, it is difficult to predict if they would be able to respond to either protection or high clipping levels.

Study Area and Treatments

Details concerning site selection and description of experiments are found in Zeigenfuss et al. (1999, this volume), and are only slightly modified here. To reduce confusion, names of sites and types of treatment follow Zeigenfuss et al. (1999, this volume). Willow communities in elk winter range of the northeast side of

RMNP were identified and random locations for potential sites were generated using a Geographic Information System (GIS). Twelve study sites were selected from these points in riparian willow communities of: (1) the north and south sides of the Moraine Park area of the Big Thompson River watershed (elevation 2,481 m), and (2) the Horseshoe Park area of Fall River watershed (elevation 2,598 m). Predominant willow species throughout all areas were *Salix monticola*, *S. planifolia*, and *S. geyeriana* mixed with other mesic shrubs, such as *Potentilla fruticosa*, *Betula glandulosa*, *B. occidentalis*, and *Alnus tenuifolia*. Eight of these sites (four in Moraine Park and four in Horseshoe Park) were located in generally drier areas (i.e., side channels, abandoned beaver dams) with little or no current beaver activity (i.e., beaver-abandoned sites) that supported short, heavily grazed willow. Evidence from dead and decadent willows on these sites indicated that plants over 2 m tall were once present and each of these sites had an abundance of evidence of prior beaver pondings (old dams, old lodges that were now abandoned). The other four sites (two in Moraine Park and two in Horseshoe Park) were located in beaver-occupied areas (including areas of recent activity) that supported taller willow plants with less severe hedging by elk, referred to as "tall willow" sites.[1] Two 30.5 m x 45.7 m paired plots were chosen at each site. One randomly selected plot was fenced to exclude elk and deer and the other remained unfenced. Exclosures were erected in August–November 1994.

In half of the sites with shorter willows (two in each drainage, n = 4 total), we attempted to raise the water table. This treatment, referred to as "short-watered willows" (sites 3, 4, 8, 9), was imposed in spring 1995. The treatments consisted of sheet metal check dams placed in both the grazed and exclosed areas at each site. The dams were placed in existing ephemeral stream channels to impede the flow of rain and snowmelt runoff and raise the water levels locally. The rest of the shorter willow sites (two in each drainage, n = 4 total) were left to their normal hydrologic regimes and are referred to as "short-control willows" (sites 5, 6, 7, 10). Sites were also classified as "wet or dry" sites based on measurements of depth to water table. "Wet" sites were the six sites with the highest average water table levels (1, 2, 4, 7, 8, 10), while those designated as "dry" sites were the six sites with the lowest water table levels (3, 5, 6, 9, 11, 12). In the results, these effects are referred to as "water table" while those designated as "tall, short-control, and short-watered" are referred to as "height/water amended" treatments.

Throughout the growing season, water table levels at all the sites were monitored using wells and by measuring water levels on either side of check dams. Annual herbaceous consumption averaged 55% of annual production and shrub consumption averaged 33% of current annual growth (27% in winter alone) throughout the study period (Singer et al., this volume). Elk were the primary ungulate herbivore in these systems.

Within each willow exclosure, simulated grazing treatments were conducted during winter (January–March) each year 1995–1998. At the outset, each exclosure was divided into sections that were randomly assigned to a "clipped" treatment or an "ungrazed" treatment. The clipped treatment, from fall 1995 onward, consisted of clipping 75% of the current year's growth from all forage shrubs and clipping 75% of the herbaceous layer in that section of the exclosure. This was intended to simulate greater herbivory that might occur with an increase in elk numbers or concentrations, and to test the effects of these higher consumption levels. All clipped plant material was removed from the exclosure. Ungrazed treatments were unmanipulated areas located within the exclosures. The unfenced plots were left to the ambient level of elk grazing of herbaceous vegetation and browsing of shrubs characteristic of that site. Elk densities and thus herbivory levels, varied greatly across these winter range sites (Singer et al., this volume). In the results, these clipping and grazing treatments are referred to as "herbivory" treatments. Twig and leaf (summer) or twig only (winter) samples were collected from the current annual growth (CAG) of the same tagged willow plants in July 1995, July 1998, and January 1999.

One 0.4 ha exclosure was established in 1963 in a willow/wet meadow community in the Beaver Meadows area of the elk winter range. Few willows were available, so we selected 15 plants from a mix of species and randomly assigned each to a clipping treatment of 0%, 50%, or 100%. During winter (prior to spring budbreak), we removed all CAG from plants clipped at the 100% level, and we systematically clipped every second CAG shoot from plants clipped at the 50% level. The 0% treatment willow plants were completely protected

[1]Two of the "short willow" sites that were beaver-abandoned in Moraine Park had high water tables. Best model selection suggested that high levels of elk herbivory at these sites were a more important factor to the willow growth parameters than was depth to the water table (Singer et al., this volume; Zeigenfuss et al., this volume).

from ungulate herbivory and clipping. We clipped willows from spring 1993 and through winter 1995–1996. All willow plants inside the exclosure had been protected from ungulate herbivory for 30 years prior to the beginning of the current study. Samples were collected in July 1996 from all 15 plants inside the exclosure, as well as from several willows (n = 10) exposed to ambient elk grazing (~33%) outside the exclosure.

Total tannins and phenolics from the current year's growth were analyzed to determine if there were differences in their production due to clipping or elk grazing, or due to artificial water amendment, or differences in water table levels. Leaf litter was collected from plants inside exclosures and those grazed by elk outside, but adjacent to the exclosures, in 1995. This was done by collecting senesced leaf material directly from willow plants prior to leaf abscission. Leaf litter that was exposed to decomposition in the field for six months was also collected as part of another study (Menezes et al. 2001) and analyzed for tannin and phenolic concentration.

Isolation and Quantification of Tannins

Shoots from each plant were analyzed for total tannins by the butanol-HCl method keeping willow species separate. Approximately 200 mg of freeze dried leaf and twig tissue (July 1995 and July 1998 samples) or twig tissue (January 1999) were extracted in 70% aqueous acetone in a test tube by sonication for 5 minutes at room temperature, and then centrifuged (4,500 rpm) for 10 minutes. The supernatant was taken to dryness, and 7 ml distilled water was added and sonicated for 5 minutes to dissolve the extract. From the solution, 0.2 ml was pipetted out of the solution into a test tube containing 4.8 ml MEOH. This was thoroughly mixed. One ml was removed and added to 6 ml butanol-HCl and 0.2 ml ferric ammonium sulfate solution. All was well shaken and heated to 95°C for 40 minutes. The sample was cooled and analyzed at 520 nm wavelengths using a spectrophotometer. From each willow species, tannins were extracted, purified, and used to formulate the reference standard for that species. Data were expressed as mg/g dry wt. Litter that was collected represented a combination of three willow species that were common at each site (*S. geyeriana, S. planifolia,* and *S. monticola*).

Isolation and Quantification of Phenolics

Freeze dried leaf and twig tissue (200 mg), or twig tissue (200 mg) for the winter sample, were extracted three times each with 3 ml $MeOH/CH_2Cl_2$ (50/50). The three extracts were combined. This solution was filtered and injected into a high-pressure liquid chromatograph (HPLC; Hewlett Packard 1090). HPLC solvents were: solvent A = water-acetic acid (98:2), solvent B = acetronitrile-acetic acid (98:2). Temperature was 50°C, flow was 1 ml/min, and wavelength detection was at 280 nm. The column was a Phenomenex Ultracarb 20, C_{18} reverse-phase 5-m column 25 cm x 4.6 mm, and fitted with a matching guard column. Total phenolics for both fresh and litter tissues were determined by summing the peak heights for compounds that consistently appeared each year in the chromatograms. These were generally the major peaks in the chromatograms. Data were expressed as relative peak height divided by 10,000 on a dry weight basis.

Statistical Analysis

All analysis was performed using SAS (ver. 6.12) statistical software. Total phenolics and tannins were tested for differences between species, water table, height/water amended, and herbivory treatments, and their interactions. Total phenolics and tannin data were analyzed for differences using the PROC MIXED program in SAS. This is a procedure that is a generalization of the standard linear model designed to analyze data generated from several sources of variation (SAS Institute 1995). Outliers were noted and eliminated from the sets according to the criteria found in the SAS univariate procedure. The number of samples analyzed for the July 1995, July 1998, and January 1999 data sets for each set of water table-height/water amended-herbivory-species combination ranged from three to five. Total sample number analyzed for July 1995 was 264, July 1998 was 235, and for January 1999, 273 samples.

The responses of willow phenolics and tannins among species, water table, and grazing and water amendment treatments were examined. Some investigators (Alstad et al. 1999; Zeigenfuss et al., this volume) found few or no differences in several willow measurements (height, annual production, rates of photosynthesis, total plant water potential) in the short willow sites due to water amendment. However, phenolics and tannins may respond differently in willows as compared to responses in willow elongation growth rates, current annual biomass production, or N dynamics. Consequently, we analyzed phenolic and tannin data in the context of the tall, short-control, and short-watered sites as well.

One of the objectives of this study was to determine if a different effect of clipping would occur on phenolic

and tannin concentration of willows that had been isolated from grazing for about 30 years, in comparison to willows that had been removed from grazing for only four years or had clipping imposed upon them consequent with protection from elk grazing. Two sources of variation that had not been addressed previously were of concern; one due to chance variation in the aliquot of sample taken from the bulk sample for analysis, and the other due to species differences. These are especially important because the sample size is small due to the limitations of only one long-term exclosure and few individuals of each species being available. The variation within an individual willow was addressed by determining the phenolic and tannin concentration on three subsamples taken from each sample. An average across the subsamples was used in the statistical analysis. The tannin data were combined across species at this long-term exclosure. However, qualitative and quantitative differences in phenolics among willow species indicated that total phenolics could not be combined across species. Thus, we reduced the samples available for the analysis of the treatment effect on phenolics to only *S. monticola.*

Additionally, a colinearity test between the ambient elk consumption rates of willows at each site and phenolic and tannin concentrations was conducted using a SAS PROC GLM program. This linear regression analysis was carried out to determine if any relationship existed between consumption rates at each site and natural product concentration. The specific pairwise comparisons were elk consumption vs. 1995 summer willow tannin and phenolic concentration, 1998 summer tannin and phenolic concentration, and 1999 winter tannin and phenolic concentration. No significant ($P < 0.05$) correlations were noted in these analyses for any year or season.

Results

Tannin Responses to Treatments in 4-Year Exclosures

July 1995 represents the first year of sampling of willows for phenolics and tannins after installation of treatments. Ungrazed and clipped willows produced more tannin than did grazed willows when species and sites were combined (Table 2b). *S. monticola* tannin production in leaves and twigs was not significantly different from *S. planifolia*, but both species had higher tannin production than *S. geyeriana* (Table 2c,d,e). As expected, willows from the tall sites produced significantly more tannin than those at the short-control and short-watered sites (Table 2c). *S. monticola* produced more tannin at the wet sites, while *S. planifolia* willows produced more tannin at the dry sites (Table 2d).

After 4 years of treatments (July 1998), ungrazed willows produced more tannin than did clipped willows, but neither of these were significantly different in tannin production compared to grazed willows (Table 3b). All species were significantly different from the other species in their tannin production with *S. monticola* producing the highest level and *S. geyeriana* the lowest (Table 3c,d). Overall, there was no significant difference in tannin production between wet and dry sites (Table 3c). However, *S. planifolia* produced more tannins in willow leaves and stems at the dry sites compared to wet sites (Table 3c).

No differences in tannin production were found between grazed, ungrazed, and clipped willows when pooled across species in January 1999 (Table 4a). There were significant differences between all species with *S. planifolia* twigs containing the highest amount of tannin and *S. geyeriana* the lowest (Table 4b,c,e). No overall differences among tall, short-control, or short-watered sites were observed, but *S. geyeriana* and *S. monticola* produced more tannin at the tall sites than at the short-control sites (Table 4b). Alternatively, *S. planifolia* willows at the short-control sites produced more tannin than did willows at the short-watered sites (Table 4b). No overall differences were observed in tannin production between wet and dry sites (Table 4c); however, *S. planifolia* produced more tannin in willows at the dry sites compared to those at the wet sites (Table 4c). Finally, ungrazed and clipped willows at the dry sites produced more tannin than did grazed willows, but no differences among grazing treatments occurred at the wet sites (Table 4d).

Phenolic Responses to Treatments in 4-Year Exclosures

The phenolic data for 1995 did not meet the criterion of homogeneity for the statistical analysis; consequently, no statistical analysis was performed on these data. Unexpectedly, more phenolic was produced by willows at the dry sites in July 1998 compared to those growing on sites with a higher water table (Table 5b). No overall differences were observed among willows at tall, short-watered, and short-control sites (Table 5c). For individual species, no differences in phenolic production across sites was noted for *S. geyeriana* and *S. planifolia* (Table 5c),

Table 2. Tannin production (mg/g dry wt) in willow on elk winter range of Rocky Mountain National Park, Colorado, July 1995. (a) Test statistics for tannin production among species, water table, height/water amended and herbivory treatments, and their interactions; (b) mean tannin production ($\bar{x} \pm$ se) among herbivory treatments when sites and species are combined; (c) mean tannin production ($\bar{x} \pm$ se) among species across tall, short-control and short-watered sites, and for species when sites are combined and sites when species are combined; (d) mean tannin production ($\bar{x} \pm$ se) among species across wet and dry site; and (e) for species when sites are combined.

(a) Source	F	*P*
Species	6.0	0.003
Height/water amended	4.6	0.010
Herbivory	2.7	0.066
Water table	0.6	0.425
Species x Height/water amended	3.0	0.018
Species x Herbivory	1.7	0.139
Water table x Species	9.6	0.000

(b) Herbivory	Ungrazed	Clipped	Grazed
	68.8 ± 2.2^{a}	69.7 ± 2.0^{a}	63.7 ± 2.0^{b}

(c)[1] Species x Height/water amended	Tall	Short-control	Short-watered
S. geyeriana	67.8 ± 3.2^{a}	52.8 ± 3.2^{b}	66.4 ± 3.7^{a}
S. monticola	75.6 ± 3.0^{a}	72.3 ± 3.1^{a}	65.3 ± 2.8^{b}
S. planifolia	73.4 ± 3.4^{a}	$73.0 \pm 7.9^{a,b}$	62.6 ± 3.1^{b}
Height/water amended	72.2 ± 1.9^{a}	65.3 ± 2.9^{b}	64.7 ± 2.5^{b}

(d)[1] Species x Water table	Wet	Dry
S. geyeriana	60.1 ± 2.9^{a}	63.9 ± 2.7^{a}
S. monticola	76.9 ± 3.0^{a}	65.4 ± 2.0^{b}
S. planifolia	63.3 ± 3.5^{a}	76.2 ± 4.0^{b}

(e) Species	*S. geyeriana*	*S. monticola*	*S. planifolia*
	62.0 ± 1.9^{a}	70.9 ± 1.7^{b}	69.5 ± 2.9^{b}

[1]Horizontal means, se followed by different letters significantly different ($P \leq 0.10$) between sites within a species.

Table 3. Tannin production (mg/g dry wt) in willow on elk winter range of Rocky Mountain National Park, Colorado, July 1998. (a) Test statistics for tannin production among species, water table, height/water amended and herbivory treatments, and their interactions; (b) mean tannin production ($\bar{x} \pm$ se) among herbivory treatments when sites and species are combined; and (c) mean tannin production ($\bar{x} \pm$ se) among species across wet and dry sites; and (d) for species when sites are combined.

(a) Source	F	*P*
Species	76.8	0.000
Water table	2.4	0.159
Height/water amended	0.3	0.746
Herbivory	3.1	0.045
Species x Water table	2.4	0.095

(b) Herbivory	Ungrazed	Clipped	Grazed
	67.4 ± 1.6[a]	62.4 ± 1.5[b]	65.0 ± 1.5[a,b]

(c)[1] Species x water table	Wet	Dry
S. geyeriana	52.5 ± 1.9[a]	53.1 ± 1.8[a]
S. monticola	77.3 ± 2.6[a]	77.4 ± 2.0[a]
S. planifolia	62.6 ± 2.5[a]	71.5 ± 2.5[b]
Water table	63.4 ± 1.5[a]	66.5 ± 1.3[a]

(d) Species	*S. geyeriana*	*S. monticola*	*S. planifolia*
	52.8 ± 1.3[a]	77.4 ± 1.6[b]	66.9 ± 1.8[c]

[1]Horizontal means, se followed by different letters significantly different ($P \leq 0.10$) between sites within a species.

Table 4. Tannin production (mg/g dry wt) in willow on elk winter range of Rocky Mountain National Park, Colorado, January 1999. (a) Test statistics for tannin production among species, water table, height/water amended and herbivory treatments, and their interactions; (b) mean tannin production ($\bar{x} \pm$ se) among height/water amended among species; (c) mean tannin production ($\bar{x} \pm$ se) between water table among species; (d) mean tannin production ($\bar{x} \pm$ se) between water table herbivory treatments; and (e) for species when sites are combined.

(a) Source	F	*P*
Species	97.9	0.000
Height/water amended	1.3	0.321
Herbivory	1.4	0.247
Water table	0.2	0.657
Species x Height/water amended	2.0	0.101
Species x water table	3.4	0.035
Water table x Herbivory	2.5	0.083

(b)[1] Species x Height/water amended	Tall	Short-control	Short-watered
S. geyeriana	54.8 ± 2.0[a]	49.5 ± 2.2[b]	50.2 ± 2.2[a,b]
S. monticola	64.1 ± 2.2[a]	58.4 ± 2.2[b]	60.9 ± 2.1[a,b]
S. planifolia	81.4 ± 3.1[a]	91.7 ± 6.4[a,b]	78.0 ± 3.1[a,c]

(c)[1] Species x Water table	Wet	Dry
S. geyeriana	53.3 ± 1.9[a]	49.6 ± 1.7[a]
S. monticola	60.2 ± 2.0[a]	62.0 ± 1.6[a]
S. planifolia	79.8 ± 3.5[a]	87.4 ± 3.1[b]

(d)[2] Water table x Herbivory	Ungrazed	Clipped	Grazed
Wet	64.8 ± 2.3[a]	62.1 ± 2.1[a]	63.6 ± 2.6[a]
Dry	65.4 ± 2.0[a]	67.4 ± 2.1[a]	60.9 ± 1.7[b]

(e) Species	*S. geyeriana*	*S. monticola*	S. *planifolia*
	51.4 ± 1.2[a]	61.1 ± 1.2[b]	83.5 ± 2.5[c]

[1]Horizontal means, se followed by different letters significantly different ($P \leq 0.10$) between sites within a species.
[2]Horizontal means, se followed by different letters significantly different ($P \leq 0.10$) between herbivory treatments within a site designated by water table.

Table 5. Phenolic production (expressed as peak height divided by 10,000, dry weight) in willow on elk winter range of Rocky Mountain National Park, Colorado, July 1998. (a) *t*-test statistics for phenolic production among species, water table, height/water amended and herbivory treatments, and their interactions; (b) mean phenolic production ($\bar{x} \pm$ se) between water table; (c) mean phenolic production ($\bar{x} \pm$ se) species x height/water amended interaction; and (d) for species when all sites are combined.

(a)

Source	F	*P*
Species	6,072.2	0.000
Water table	6.8	0.044
Height/water amended	0.5	0.639
Herbivory	1.7	0.177
Species x Height/water amended	0.2	0.051

(b)

Water table	Wet	Dry
	1,583.8 ± 70.5[a]	1,833.7 ± 67.0[b]

(c)[1]

Species x height/water amended	Tall	Short-control	Short-watered
S. *geyeriana*	72.9 ± 4.3[a]	70.4 ± 5.5[a]	83.9 ± 5.8[a]
S. monticola	3,221.4 ± 175.1[a]	2,720.8 ± 170.2[b]	2,850.6 ± 166.9[a,b]
S. planifolia	23,495.6 ± 1438.9[a]	23,148.1 ± 2,784.7[a]	20,685.1 ± 362.3[a]

(d)

Species	*S. geyeriana*	*S. monticola*	*S. planifolia*
	75.5 ± 3.0[a]	2,923.0 ± 98.8[b]	22,414.7 ± 1121.2[c]

[1]Horizontal means, se followed by different letters significantly different ($P \leq 0.10$) between sites within a species.

but *S. monticola* produced more phenolic compounds at the tall site compared to willows at the short-control site.

Phenolic production in 1999 winter willow twigs differed between species with *S. geyeriana* producing significantly more phenolics in twigs than either of the other two species (Table 6b). *S. planifolia* produced the least quantity of phenolic compounds (Table 6b). Clipped willows produced significantly more phenolic than did ungrazed or grazed willows (Table 6c). Phenolic production among willows at the dry sites, when species and treatments were combined, was higher than that for willow at the wet sites (Table 6d).

Phenolic and Tannin Production in Willows Protected from Grazing for 35 Years

For the 35 year-old exclosure, tannins were significantly higher in the 50% clipped treatment than the 0% clipped, 100% clipped, or elk herbivory treatments (Table 7). Tannins from willows subjected to the 100% clipping level inside the exclosure were lower than the tannin concentration in ungrazed (0% clipped), lower than in the 50% clipped treatment, and lower than in willows outside the exclosure grazed by elk. Total phenolics increased in the 50% clipped treatment ($P = 0.155$; Table 7). Response of willow height and growth paralleled the responses for tannins and phenolics. Clipping willows at the 50% level resulted in no reductions of rate of height growth or rate of CAG production of willows (F = 0.21, F = 0.93 respectively; $P = 0.81$, $P = 0.4$ respectively; Singer et al. [1998]).

Willow Litter Phenolics and Tannins

Decomposed litter collected from tall, short-control, and short-watered sites contained only a minor amount of phenolic, and no measurable tannin, when compared to fresh litter (Table 8). Fresh litter contained higher tannin and phenolic concentrations than decomposed litter at all sites ($P < 0.05$). However, no differences were found between treatments within a site.

Table 6. Phenolic production (expressed as peak height divided by 10,000, dry weight) in willow on elk winter range of Rocky Mountain National Park, Colorado, January 1999. (a) Test statistics for phenolic production among species, water table, height/water amended and herbivory treatments, and their interactions; (b) mean phenolic production ($\bar{x} \pm$ se) among species when all treatments combined; and, (c) mean phenolic production ($\bar{x} \pm$ se) among herbivory when species and water table and height/water amended are combined; and (d) mean phenolic production ($\bar{x} \pm$ se) for water table when species and treatments are combined.

(a) Source	F	*P*
Species	215.1	0.000
Height/water amended	0.8	0.493
Herbivory	2.5	0.090
Water table	8.3	0.025
Species x Water table	1.9	0.154

(b)[1] Species	*S. geyeriana*	*S. monticola*	*S. planifolia*
	140.3 ± 4.7[a]	130.9 ± 3.7[b]	57.4 ± 2.1[c]

(c)[1] Herbivory	Ungrazed	Clipped	Grazed
	100.0 ± 3.3[a]	107.3 ± 3.4[b]	98.3 ± 3.2[a]

(d)[1] Water table	Wet	Dry
	95.8 ± 3.1[a]	108.2 ± 2.9[b]

[1]Horizontal means, se followed by different letters significantly different ($P \leq 0.10$).

Discussion

Species Differences

Species differed in phenolic and tannin production in practically every comparison regardless of treatment or how sites were divided. In particular, tannin production in summer twigs and leaves among species was highest for *S. monticola* followed by *S. planifolia* and then *S. geyeriana*, but occasionally *S. planifolia* had higher tannin concentration than *S. monticola*. Tannin production in the winter was generally highest for *S. planifolia* followed by *S. monticola* and then *S. geyeriana*. A similar pattern was observed for phenolic production in summer twigs and leaves among species with *S. planifolia* highest followed by *S. monticola* and then *S. geyeriana*. For winter twig samples, phenolic production was highest in *S. geyeriana* followed by *S. monticola*. Phenolic production in samples of summer twigs and leaves combined was significantly greater than that of twigs collected in the winter.

A high level of variation in total phenolic production was shown among the three species tested. This variation is primarily due to each willow species differing in their qualitative and quantitative production of phenolics. Interaction between variables was apparently species driven (Table 6). This source of variation in itself may have masked water table, height/water amended, and herbivory effects. In addition, *S. monticola* and *S. planifolia* did not respond to water table and herbivory effects in the same way. For example, tannin production in 1995 was higher for willows of *S. monticola* at the wet sites, but for *S. planifolia* tannin, concentration was always higher in willows of this species growing on drier sites (Tables 2d, 3c, 4c).

Phenolic and Tannin Production in Willows as Related to Water Table and Treatments

It is unclear as to why a lack of homogeneity occurred in the July 1995 phenolic data set. No other data set had a similar problem. This collection immediately

Table 7. Total tannin (mg/g dry weight) response [$\bar{x}$ (sd)] and phenolic (expressed as peak height divided by 10,000, dry weight) in willow species subjected to clipping levels inside and outside of 35-year old exclosures on elk winter range in Rocky Mountain National Park, Colorado, July 1996.

	Simulated herbivory			Elk herbivory
Species	0%	50%	100%	
All species				
Tannins*	60.9(10)[a]	80.1(17)[b]	48.7(2)[c]	63.1(22.5)[a]
S. monticola				
Phenolics**	297(35)[d]	411(87)[e]	307(17)[#]	337(81)

*Means followed by different letters are significantly different, $P < 0.10$.
** Phenolics for *S. monticola* only; means followed by the letters d,e; $P = 0.155$ (n = 2 or 3).
[#] sd is the $\bar{x}$ of three runs of the same sample for the only individual in this category (not used in statistical analysis).

Table 8. Tannin (mg/g dry weight) and phenolic (expressed as peak height divided by 10,000, dry weight) concentration of fresh willow litter versus decomposing willow litter among herbivory and height/water amended treatments [$\bar{x}$, (sd*)] on elk winter range in Rocky Mountain National Park, Colorado, 1995. No significant differences between treatments were found.

Height/water amended treatment		Ungrazed	Clipped	Grazed
Tall				
Phenolics	F	424 (162)	555 (38)	412 (206)
	D	2,011 (501)	3,326 (538)	2,913 (2,693)
Tannin	F	64.1 (21)	67.7 (10)	65.1 (19)
Short-control				
Phenolics	F	189 (33)	372 (283)	325 (269)
	D	50 (42)	29 (98)	21 (9)
Tannin	F	77.2 (8)	70.7 (2)	72.0 (4)
Short-watered				
Phenolics	F	NA	NA	NA
	D	40 (26)	22 (9)	22 (8)

*All comparisons between fresh and decomposed litter significantly different, $P < 0.05$.
NA = data not available; F = fresh litter; D = decomposed litter.

followed the beginning of the clipping treatment and perhaps the initial clippings disrupted natural product metabolism such as was noted for Douglas-fir phenolic and tannin metabolism (Horner et al. 1993). In addition, the weather in spring 1995 was unusually cold and wet. Standing water from spring runoff was present on many sites into July, and cold temperatures delayed both spring runoff and leaf emergence 3–4 weeks (Zeigenfuss et al., this volume; L. Zeigenfuss, personal observation).

With regard to site quality, the expectation was that tall willow growing on sites with some beaver activity would produce the highest levels of phenolics and tannins. The data supported this expectation. Tannin production in willows at the tall sites in 1995 was higher compared to the short-control and short-watered sites. Tall willows from *S. geyeriana* and *S. monticola* in January 1999 also had generally higher tannin concentration compared to willows on the short-control sites, but tall willows did not differ significantly from those at the short-watered sites.

The tall willow with some beaver activity category represented the accumulated product of multiple factors in Rocky Mountain NP. The height of the willows was primarily influenced by elk densities and rates of elk herbivory (Singer et al., this volume). Multiple factor models and information criteria suggested that elk herbivory was the factor of overriding importance on eight parameters of willow growth and thus on observed willow heights, and that depth to the water table had a significant, but lesser, influence (Singer et al., this volume). Elk rate of consumption of CAG of 37% had a negative, suppressing influence on willow growth and biomass production peaked at consumption levels of about 21% (Singer et al., this volume). In other words, the tall willow patches were tall primarily due to elk herbivory levels, which were lower than for the short (beaver-abandoned) willow patches, but shallower depth to the water table and beaver dams also contributed to larger, more optimal sizes of willows. Other associated factors also contributed to the large size and higher densities of willow stems in this type. A portion of the willow plants were above the reach of elk and thus unbrowsed each year, the dense jungle of willow clumps also protected part of the patch from access by elk, and any active beaver dams and ponds made some of the willows less accessible to elk. Beaver dams increased the height of the water table and ponds neglected for a few years by beaver still retained some water. Also, depth of the rooting zone of short browsed willows on sites away from streamsides were apparently more shallow, further contributing to less-than-ideal water relations of the short-control willow category. Although depth to the water tables was shallow for a few of the short control patches, this type was generally characterized by long-term beaver abandonment of the site. There were no active dams, and streamside channels, meanders, oxbows, and braided channels were all dry, except for brief periods in the spring during peak run-off.

The short willows (beaver-abandoned sites) produced less secondary metabolites in RMNP (and also in Yellowstone NP; Singer et al. [1994], Singer and Cates [1995]) and these lower levels were near or below levels (e.g., 5% for tannins) that have been identified as thresholds where ungulates will browse more on shrubs (Cooper and Owen-Smith 1985). The lower growth rates, poorer growing conditions, and possible greater palatability to ungulates place the short willows in a downward spiral, particularly if elk populations continue to increase on the winter range. Apparently, long-term responses of heavily browsed willows led them to be more vulnerable to ungulate herbivory. This vulnerability includes the entire willow plant becoming within accessible reach of ungulates through shorter stature and smaller canopies with more perimeter accessible to browsers, lower growth rates, and lower secondary metabolites leading to greater palatability.

When sites were designated as wet or dry sites based on water table levels, mixed results occurred. In some cases, phenolics or tannins were higher in willows on the wet sites, but in other cases, these compounds were higher in concentration in willows on dry sites. Overall, the tall-short willow designation appeared to represent site quality better compared to the designation by water table depth (wet vs. dry).

We expected that willows would respond to both clipping and grazing through an increase in willow phenolic and tannin. The January 1999 clipped willows showed a significant increase in phenolics compared to ungrazed willows. However, in the majority of samplings, no difference in response in either phenolics or tannins occurred between the clipping and grazing treatments. Either the willows in general were not growing on sites favorable for supporting vigorous growth and hence were unable to respond to clipping or grazing, or the clipping and grazing levels were too severe. Data from willows in the 35-year-old exclosure and other studies on the willows at the study sites (Singer et al., this volume), suggest that the latter was likely and that ambient levels of elk herbivory not only negatively influence a wide spectrum of willow growth

parameters (total CAG, twig CAG, mean height, maximum height stem density, plant density, canopy volume and area, catkin production; Singer et al., this volume; Zeigenfuss et al., this volume), but also production of secondary metabolites. The data from the 35-year exclosure also may indicate that 30 years relief from grazing allowed willows to recover enough to respond chemically to moderate levels of clipping.

Response of Tannins and Phenolics to Treatment in the 35-Year-Old Exclosure Compared to the 4-Year-Old Exclosure

The expected response was that the 50% clipping would result in an induced increased production in tannins and phenolics. Our observations in the 35-year-old exclosure support this expectation--tannins increased under 50% clipping and decreased significantly in the 100% clipping level compared to the no clipping treatment. With regard to elk grazing, the decreased response in tannins in willows grazed by elk outside the long-term exclosure suggests that elk have affected the ability of willows to respond to grazing. The significant increase in tannins at the 50%, but not at the 100% simulated herbivory level is also consistent with the literature (Karban and Baldwin 1997), and suggests that growing conditions inside this exclosure were sufficient to allow willows to respond positively in terms of secondary metabolites to this moderate clipping level.

The responses in the 4-year exclosures suggested that the 75% clipping was too high to permit an induced reaction in willows. Total phenolic production was lower in the older exclosure than observed for *S. monticola* in the 4-year-old exclosure possibly because willows inside the older exclosure had not been grazed for an extended time. The evidence also suggests that the 75% clipping level was too severe for a willow response in the 4-year-old exclosure, particularly with no recovery period from grazing.

It was expected that willows outside the exclosure would also respond to elk grazing by increasing their secondary metabolite production such as that observed for the 50% clipping. But we observed that elk grazed willows did not respond by increasing secondary metabolite production suggesting that elk herbivory levels had an adverse, long-term, accumulated, negative effect on not only willow size, structure, depth to the rooting zone, physiology, and growth rates (Alstad et al. 1999; Menezes et al., this volume; Singer et al., this volume; Zeigenfuss et al., this volume), but also a negative effect on the ability of willow to respond to herbivory with increased production of secondary metabolites. Overall, the pattern noted among treatments inside the 35-year-old exclosure represents the expected response of willows that are growing in a favorable environment.

Information from Yellowstone NP (YNP) tends to corroborate these conclusions. Apparently, most willows are growing on poor sites on Yellowstone's northern winter range (Singer et al. 1994) and the growing conditions are less favorable than in RMNP (Singer et al. 1998). Similar clipping experiments in YNP revealed no response in tannin or phenolic production at the 50% clipping level, and significant decreases in heights (22% decline) and CAG (250% decline) at the 100% clipping level (Singer et al. 1998). Similarly, height-suppressed, ungulate-browsed willows in YNP produced 41–64% less phenolics and 15–17% less tannins than ungrazed controls (Singer et al. 1994; Singer and Cates 1995), declines due to ungulate herbivory that are unprecedented in the ecological literature. The evidence from both parks suggests that poor site growing conditions and a long history of heavy browsing with associated reductions in plant sizes (and possibly also root biomass and depths) reduces the ability of willows to respond to ungulate herbivory by increasing production of secondary metabolites. Declines in nitrogen concentrations and content also occurred at the heaviest clipping levels (100%) and in ungulate-browsed willows compared to controls in YNP (Singer et al. 1998). Tannin production may be linked to the availability of nitrogen.

Summary

Based on our conceptual review of prior research of the effects of growing conditions and grazing on willow chemistry, the expected results were that willows growing on favorable habitats would respond to clipping and elk grazing by increasing their phenolic and tannin concentration. Patterns across the three years of sampling of the current year's growth in a series of 12 exclosures in place for only 4 years were not consistent with these predicted results probably because the clipping treatment of 75% was too severe to permit an increase in phenolic and tannin concentration. However, results at an exclosure in place for 30 years were consistent with the expectations, and willows clipped at moderate levels (50%) responded by increasing phenolic and tannin

content, while willows clipped at high levels (100%) and unclipped willows (0%) did not. Tall willow sites with less negative effects of elk herbivory on size and growth of willow, and with beaver activity (cuttings, some active dams) and the presence of standing water in channels, followed our original expectation and possessed higher concentrations of phenolics and tannins compared to short, hedged willows growing on beaver-abandoned sites (dry channels, abandoned dams). Few significant increases in phenolic and tannin concentration were noted in the clipping and grazing treatments, apparently because there has been a long-term (elk have been at high densities at the study sties for about 20 years) negative effect of the ambient levels of elk herbivory (average 33% removal of CAG) on willow response, and apparently because the 75% clipping treatment was too severe to permit an increased response by the willows. The lack of response in willows subjected to natural grazing levels, suggest that elk grazing has adversely affected the ability of willows to respond chemically to grazing and these lower levels of secondary metabolites can lead to greater palatability of grazed willows. Increased grazing pressure from increasing populations or concentrations of elk could further adversely affect willow growth and nutrient dynamics in these communities.

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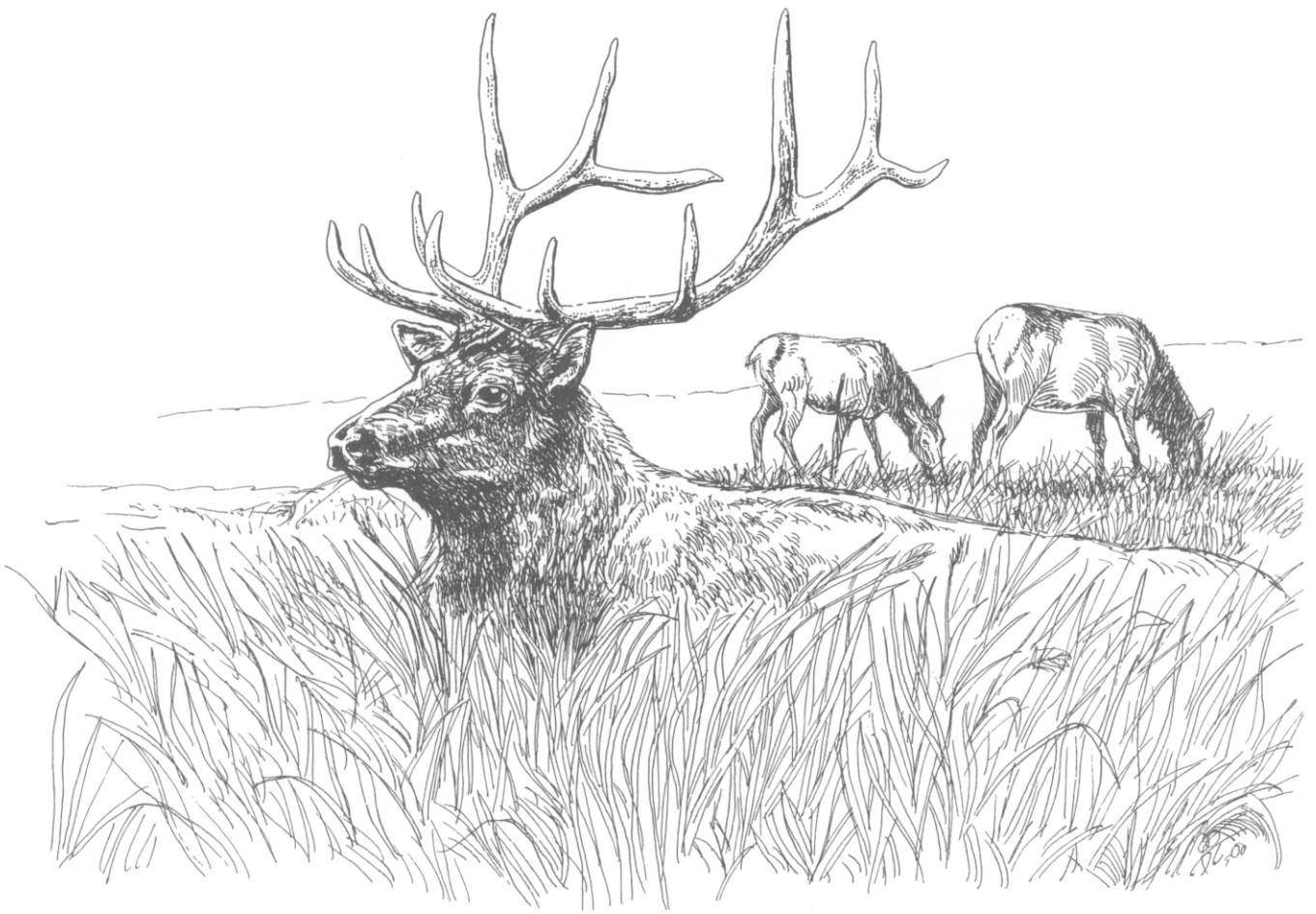
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